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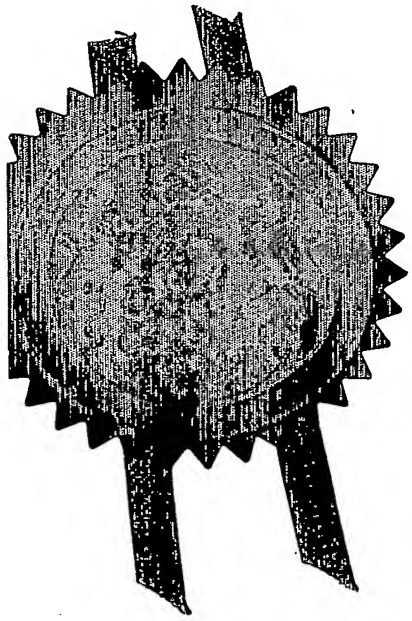

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12NOV02 E762649-1 D02866
P01/7700 0.00-0226275.6

2. Patent application number *(The Patent Office will fill in this part)* — 0226275.6

12 NOV 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Royal Veterinary College
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United Kingdom

Patents ADP number *(if you know it)* 8504177001

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention

METHOD OF TREATMENT

5. Name of your agent *(if you have one)*

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"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Patents ADP number *(if you know it)* 1305010

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

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a) *any applicant named in part 3 is not an inventor; or*
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11. I/We request the grant of a patent on the basis of this application.

Eric Potter Clarkson

Signature: **ERIC POTTER CLARKSON** Date: 12 November 2002

12. Name and daytime telephone number of person to contact in the United Kingdom: **0115 9552211**

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METHOD OF TREATMENT

The present invention relates to a method of treatment of soft skeletal tissue injury in a patient; in particular, it relates to the treatment of tendon or ligament injuries particularly but not exclusively in competitive or racing mammals such as humans, horses, dogs and camels.

Superficial digital flexor tendon injuries are a common cause of wastage amongst competition horses, associated with a poor success for a return to a previous level of performance and a high incidence of re-injury. Current treatment regimes (reviewed by Dowling *et al*, 2000) have only marginal effects on the outcome of tendinopathy with the major influence on prognosis being the severity of the initial injury. Recent studies investigating the efficacy of the lysyl oxidase inhibitor, beta-aminoproprionitrile fumarate, demonstrated significant improvements in outcome for moderate to severe superficial digital flexor tendinopathy (Genovese, 1992), although this has not been so favourable in further clinical trials (Reef *et al*, 1996, 1997) and recent experimental work has demonstrated possible adverse effects of this treatment (Dahlgren *et al*, 2002). Furthermore, while this treatment prevents collagen cross-links forming too early thereby allowing a controlled exercise regime to improve the functionality of the scar tissue, it does not regenerate tendon tissue. As scar tissue will never be as functional as tendon tissue, a goal of future efficacious treatment is to develop methods of regenerating tendon tissue.

There has been considerable interest recently in the potential therapeutic benefits of mesenchymal stem cells (MSC) for tendon and ligament healing (Woo *et al*, 1999; Caplan and Bruder, 2001; Hildebrand, *et al*, 2002). These cells reside in small numbers in all tissues and possess multipotential capabilities of differentiating into a number of different tissues. Recent

reports have shown that MSCs can be implanted into tendon and ligament tissue using scaffolds in experimental animals (Young *et al*, 1998). One source of the MSCs has been bone marrow, and recent reports (Herthel, 2001) have reported considerable success in the use of bone marrow aspirated from the sternebrae and injected direct into the damaged tendon or ligament. In this report, the overall prognosis for a return to full work in 100 horses with suspensory ligament injuries, treated with bone marrow was 84%, while a comparative group of 66 horses treated conservatively had a prognosis of 15%. However, there is no documentation of the numbers of forelimb and hindlimb injuries, nor the region of the suspensory ligament damaged, all of which are known to have very different prognoses (Dyson, 1995, 2000). Furthermore, this technique has many limitations. Injection of large volumes of bone marrow (30-50 ml) would potentially cause considerable disruption of the remaining intact tendon tissue, would include other components of bone marrow such as bone spicules, fat cells, etc deleterious to tendon healing, and only small numbers of MSCs would be expected to be present. Neither the presence of or number of mesenchymal stem cells in this method of treatment have been described or validated. Some clinicians have thus doubted the efficacy of this technique as smears of aspirated bone marrow resemble peripheral blood smears.

In a preferred embodiment of the present invention a technique has been developed for the isolation, characterisation, and expansion *in vitro* of equine MSCs, with re-implantation of large numbers of autologous MSCs into a damaged superficial digital flexor tendon in the horse. MSCs have the potential to differentiate into tenocytes and regenerate tendon matrix after injury.

The invention is not limited to the treatment of horses nor, indeed, to the treatment of the superficial digital flexor tendon or the use of autologous

cells, although this is preferred. Rather, it has wider applications as is herein described in detail.

A first aspect of the invention provides a method of treating a natural soft skeletal tissue injury in a patient the method comprising administering to the patient a composition of mesenchymal stem cells in liquid suspension enriched compared to the natural source of said cells.

Soft skeletal tissue includes tendons, ligaments, intervertebral discs, which are associated with spinal pain or injury, and menisci.

Soft skeletal tissue can be injured in various ways, such as by surgical laceration which is a type of percutaneous traumatic injury. Such surgical injuries may be considered to be "unnatural". The injuries for treatment by the present invention are "natural" injuries by which we mean the injury typically occurs subcutaneously, for example by way of being strain induced, which is often an accumulation of damage over a period of time. Thus, natural injuries are clinical injuries and include traumatic injuries that present to the clinician, including accidental lacerations.

Such natural injuries are common in competitive or racing animals, including humans. Natural soft skeletal tissue injuries can readily be diagnosed by the physician or veterinary surgeon using well known techniques such as considering the patient's history, clinical examination, palpation, ultrasound examination, MRI scan and the like.

It is preferred that the injury to be treated is a strain injury.

It is preferred if the soft skeletal tissue that is treated is a tendon or ligament. Particularly preferred tendons or ligaments for treatment by the

method of the invention are those that are commonly injured in competitive or racing or athletic animals by strains or an accumulation of damage, such as strain damage.

The patient may be any suitable patient. Typically the patient is a mammal (by which we include humans). Typically, the non-human animal such as a non-human mammal is one of economic importance, such as a racing animal or working animal. Even more typically the animal is a mammal which undergoes competition (ie sporting competition), such as a human, horse, dog (such as whippets, greyhounds, gun dogs, hounds, huskies) or camel.

It is particularly preferred if the patient is a horse which, because of their use in sports (racing, jumping, showing etc), or as work animals, they are particularly susceptible to natural injury to the soft skeletal tissue as defined.

Although in these mammals, any soft skeletal tissue injury can be treated by the method of the invention, particular injuries may more suitably be treated than others. Thus, when the patient is a horse or a camel, it is preferred if the soft skeletal tissue is selected from the group consisting of superficial digital flexor tendon (SDFT), suspensory ligament and deep flexor tendon (in both forelimbs and hindlimbs), accessory ligament of the deep digital flexor tendon (DDFT), menisci, and other ligaments such as the cruciate ligaments. When the patient is a dog, it is preferred if the soft skeletal tissue injury is selected from the group consisting of Achilles tendon, cruciate ligament, meniscus and flexor tendon. When the patient is a human it is preferred if the soft skeletal tissue selected from the group consisting of Achilles tendon, quadriceps tendon, rotator cuff, lateral or medial epichondylitis, cruciate ligament, intervertebral disc and meniscus.

The method is particularly suited to treating flexor tendons rather than extensor tendons. Flexor tendons store energy and accumulated damage that precedes partial or total rupture. Flexor tendons generally do not heal well and injuries thereto have a high morbidity.

It is particularly preferred if the method is used to treat injured tendons or ligaments which store mechanical energy. Thus, treatment of tendinitis (tendonitis), tendinopathy (tendonopathy ie injury to tendon), desmitis (injury to a ligament), bowed tendon, bowed leg and strain injuries is specifically contemplated.

The composition of mesenchymal stem cells may be any suitable composition of such cells provided that the composition is enriched compared to a natural source of said cells. Natural sources of mesenchymal stem cells include bone marrow, peripheral blood and umbilical cord, but also includes fat and muscle and, in small number, cells into which they differentiate (eg tendon, ligament, etc). The composition of cells for use in the invention may be enriched compared to the natural sources by any suitable method, typically involving cell fractionation and concentration methods. Suitable methods are well known in the art and include the Ficoll-Paque methodology described in the Example. Other suitable methods include concentration of mesenchymal stem cells using antibodies directed to mesenchymal stem cell markers which are immobilised, for example in an affinity chromatography column or to a substratum in a "panning" scheme. Enrichment can also be achieved by culturing the cells and expanding the cells under conditions which retains their character as a mesenchymal stem cells. Such methods are well known in the art, and one of those is described in detail in the Example. Mesenchymal stem cells are characterised by multipotency, ie their ability to differentiate into various

skeletal and connective tissue cell lines when appropriate biological and/or mechanical signals are present. In particular, mesenchymal stem cells are able to differentiate into cartilage, bone, muscle (such as myotubes), tendon producing cells (tenocytes), fibroblasts and adipocytes (fat producing cells). Suitably, in the enrichment process (including expansion of cells in culture), the presence of (and enrichment of, including expansion in culture) the mesenchymal stem cells can be determined prior to their use in the method of the invention by making the cells differentiate into the different cell lines characteristic for mesenchymal stem cells. Additionally or alternatively, markers (typically cell surface markers) may be useful in the identification of mesenchymal stem cells. In some species, mesenchymal stem cells exhibit the STRO1 marker (but probably not in the horse). Typically, mesenchymal stem cells produce collagen type I and COMP (see below). The gene "scleraxis" may be a marker for a tenocyte.

Typically, the enrichment of mesenchymal stem cells in the composition is at least 2-fold over the said cell content in the natural source from which they are enriched. Preferably, the enrichment is at least 3-fold, 4-fold, 5-fold, 10-fold, 20-fold or more preferably at least 30 or 40 or 50 or 100-fold.

Preferably, it is at least 1000-fold or 10^4-fold or 10^5 fold.

Typically, the enriched composition contains at least 10% of its cells as mesenchymal stem cells and preferably at least 50% or 60% or 70% or more. It may be advantageous for at least 90% or at least 95% or 99% of the cells in the composition to be mesenchymal stem cells.

It is particularly preferred if the mesenchymal stem cells are derived from bone marrow. It is particularly preferred if the cells are enriched compared to bone marrow, for example using the methods described in the Example

or variants of the method based on the general principles of cell enrichment, expansion (if necessary) and screening.

Although it is envisaged that any composition of mesenchymal stem cells enriched compared to their natural source would be useful, it is preferred if the cells are allogenic (ie from the same species as the patient), as opposed to xenogenic (ie from a different species). If the cells are allogenic, but not autologous, it is preferred if the cells are of a similar tissue type (eg have similar MHC/HLA haplotypes). It is particularly preferred if the cells are autologous (ie are derived from the patient to which they are administered). Such autologous cells have the advantage of being much less prone to rejection compared to other allogenic (or xenogenic) cells. Also, the use of autologous cells avoids any issue of "doping" (eg with "foreign" DNA) which may be of concern. Thus, a particularly preferred method of the invention comprises obtaining mesenchymal stem cells from the patient (for example from the bone marrow), enriching the cells and, if necessary, expanding them in culture, and introducing the so-enriched cells into the patient. It will be appreciated that some of the cells may be saved for use at a later date, and typically such cells are frozen under conditions that retains their viability. It will be appreciated that the cells may be obtained and enriched (expanded if necessary) before any injury to the patient, and kept for immediate administration if and when the patient sustains an injury to the soft skeletal tissue. This procedure means that no time would be lost in starting treatment following injury.

By liquid suspension of cells we include any suitable liquid suspension. For example, the liquid suspension may be a suspension of cells in a medium that contains appropriate biological signals to encourage the differentiation of the mesenchymal stem cells into cell types that are useful to the regeneration of soft skeletal tissue injuries (eg tenocytes in the case

of regeneration of tendons), and discourage the differentiation of the cells into cell types that are not useful (eg bone tissue). A suitable liquid suspension is wherein the mesenchymal stem cells are suspended in platelet rich plasma such as descried in the Example. Suitable liquid suspending media include serum, plasma, platelet rich plasma, bone marrow supernatant, or enriched or conditioned medium. For the avoidance of doubt, the liquid suspension may be one which gels *in situ*, for example because of the temperature at the injury site of the patient, or because it is mixed with another agent that causes gelling.

Suitable biological signals include molecules that encourage the cells to differentiate in the appropriate way. Such molecules may include growth factors, cytokines which, because of the very high degree of similarity between species need not be autologous or allogenic (eg human growth factors or cytokines may be used in the horse). Suitable growth factors may include TGFβ (preferably isoform 3), IGF 1, IGF 2, PDGF and FGF. It may also be useful to have present in the liquid suspension of cells, other factors that encourage the cells to regenerate as soft skeletal tissue, such as cartilage oligomeric matrix protein (COMP), which may help in soft skeletal tissue formation, but which is often not present in older animals in some species such as the horse. It will be appreciated that although it is preferred if the biological signals are present in the liquid suspension, they may alternatively or additionally be administered separately to the patient, for example at the site of injury. It is particularly preferred that the biological signals or combination thereof used are ones that reduce the possibility of scar formation.

The composition of mesenchymal stem cells is administered to the patient in any suitable way. Preferably, the composition is administered directly at the site of injury (or adjacent thereto), and typically such that the

mesenchymal stem cells remain at the site of injury. Certain sites of natural soft tissue injury comprise enclosed cavities and it is particularly preferred if the natural soft tissue injury site that is treated is one with such a cavity or a lesion that can readily be closed to form a cavity. When such injuries are treated, the chance of leakage away from the injured site is reduced. It is preferred that the injury treated is an intratendinous partial rupture. In SDFT in horses the lesion along the tendon is commonly in the mid-metacarpal region. In DDFT the lesion is often within the digital sheath. In some cases, the injury is such the damage (eg a tear) opens any cavity, or the injury is at a site where naturally there is no cavity, it may be necessary to introduce packing (for example in the form of a gel matrix), close the cavity or otherwise retain the cells at the site of the injury. Torn tendons or ligaments may be closed surgically to form a cavity into which the liquid suspension of cells may be administered. Alternatively, the composition of mesenchymal stem cells may be introduced in the torn tissue and sutured without creation of a specific cavity.

It is preferred if the composition of mesenchymal stem cells in liquid suspension is injected into the site of injury. Typically, this is by percutaneous injection, with or without ultrasound to guide the injection to the site of injury (eg within the cavity of a tendon that has been injured). Thus, the needle of a syringe may pass through the skin, straight into the soft skeletal tissue such as a tendon. Suitably, there may be a "stop" on the needle which means that its end is at the desired position within the site of injury for the release of the composition containing the cells. Alternatively, the needle for injection may be guided arthroscopically, which may constitute a minimally invasive way of getting into the soft skeletal tissue such as tendon. Arthroscopic guidance may be particularly useful where the site of injury is intra-articular or intrathecal (ie within a tendon sheath) or intra-articular collagenous structure, cruciate ligament or meniscus.

In one embodiment of the invention, the site of injury is cleansed of damaged tissue and early repair scar tissue that may be starting to form at the site before administration of the composition of mesenchymal stem cells. In this way, it may be possible to prevent or reduce the chances of scar tissue, or other undesirable tissue, formation. This may be done using minimally invasive surgical debridement, or using enzymatic or biophysical methods.

The dose of cells that is administered to the patient may vary by reference to the type and severity of the injury, and may be determined by the physician or veterinary surgeon. Typically, the liquid suspension is administered in about 0.1 ml aliquots (or 0.2 ml or 0.3 ml or 0.4 ml but typically no more than 0.5 ml aliquots) at the site of injury. Typically, an aliquot, such as a 0.1 ml aliquot, contains from about 50 000 to 500 000 mesenchymal stem cells.

The size and/or number of aliquots may vary depending on the nature and extent of the injury. The volume of the lesion (site of injury) may be accurately determined by ultrasonography. The volume of the lesion can generally be determined from the ultrasound pictures alone. Typically, when the injury is at a site which has a cavity (or can be made to form a cavity by packing as described above), the cavity is filled with the liquid suspension of cells).

It is preferred that the treatment regimens of the invention starts as soon as possible after injury; however, it may be advantageous to allow the blood to clot and start to form early granulation tissue to induce or enhance the blood supply. Desirably, treatment starts within 24 hours to 4 weeks, typically within 48 hours to 7 days.

In a preferred embodiment of the invention, the regeneration of the soft skeletal tissue at the site of injury is monitored by ultrasonography including the measurement of, cross-sectional areas (eg by ultrasonography). It is also particularly preferred that following the treatment the patient is subjected to a rehabilitation procedure involving exercise of the injured site. Typically, if the cross-sectional area of the damaged tissue (such as tendon or ligament) increases by more than 10% at any level, exercise is reduced, whereas if it remains constant or decreases, exercise is gradually increased. Suitable rehabilitation (eg exercise) regimens can readily be devised by the physician or veterinary surgeon having regard to the nature of the injury, the treatment thereof and the progress made by the patient in regenerating suitable soft skeletal tissue at the site of injury. A suitable rehabilitation regimen is shown for the treatment of SDFT in the horse.

A further aspect of the invention provides the use of a composition of mesenchymal stem cells in liquid suspension enriched compared to the natural source of said cells in the manufacture of a medicament for treating a natural soft skeletal tissue injury in a patient.

A further aspect of the invention provides a kit of parts comprising (1) a composition of mesenchymal stem cells in liquid suspension enriched compared to the natural source of said cells, (2) means for delivering the liquid suspension of stem cells to a site of natural soft skeletal tissue injury in a patient and (3) means for determining that the means for delivering locate to the site of injury.

The invention will now be described in more detail by reference to the following non-limiting Figures and Examples.

Figure 1 shows equine mesenchymal stem cells adhering to plastic after semi-purification from bone marrow by Ficoll centrifugation.

Figure 2 shows ultrasonographs of the superficial digital flexor tendon of an 11 year old polo pony with a superficial digital flexor tendonitis of 5 weeks duration prior to stem cell implantation and 10 days after stem cell implantation. The lesion occupies the central 45% of the tendon and is filled with granulation/young fibrous tissue. There has been no significant disruption to the healing tendon by the implantation procedure.

(a) Transverse image from level 4 (20 cm distal to the accessory carpal bone).

(i) Before implantation.

(ii) 10 days after implantation.

(b) Longitudinal image - 20-24 cm distal to the accessory carpal bone.

(i) Before implantation.

(ii) 10 days after implantation.

Example 1: Isolation of equine mesenchymal stem cells from bone marrow and their implantation into the superficial digital flexor tendon as a potential novel treatment for superficial digital flexor tendinopathy

Materials and Methods

Case details

Autologous MSCs were re-implanted after expansion *in vitro* into a damaged superficial digital flexor tendon of an 11 year old polo pony that had suffered a strain-induced injury of its superficial digital flexor tendon 5 weeks previously.

Bone marrow aspiration:

After sedation (with 10 µg/kg detomidine hydrochloride[1] (Domosedan, Pfizer Animal Health, Ramsgate, Kent) and 20 µg/kg butorphanol (Torbugesic, Fort Dodge Animal Health, Southampton, UK)), an area 5 cm x 20 cm over the sternum was prepared by clipping and scrubbing. After aseptic preparation, the intersternebral spaces, easily identified by diagnostic ultrasonography, were marked on the skin using a sterile marker pen. Local anaesthetic solution (2 ml; mepivicaine (Intra-Epicaine, Arnolds, Shrewsbury, UK)) was infiltrated subcutaneously over the midpoint in the sagittal plane of two adjacent sternebrae. A stab incision with a number 11 scalpel was made through the skin. A Jamshidi biopsy needle (11G, 4 inch) was introduced approximately 4-6 cm until it contacted the sternebra. It was then pushed a further 3-4 cm into the sternebra and then 1.8 ml aliquots of bone marrow from each of two sternebrae was aspirated into 2 ml syringes, pre-loaded with 1000 iu heparin (Heparin

(Multiparin, CP Pharmaceuticals, Wrexham, UK); 5000 iu/ml). Five aliquots were taken in the first series of aspirates to quantify MSCs cell numbers and thereafter 2 aliquots were taken for preparation of MSCs for re-implantation. The 1.8 ml marrow aspirates were gently oscillated and then transferred into sterile 5 ml tubes and placed on ice for immediate transfer to the laboratory.

Mesenchymal stem cell isolation and *in vitro* culture and expansion:

The MSCs were separated using a technique similar to that described for the isolation of MSCs in other species (Rickard *et al*, 1996). In brief, the initial 2 ml of a bone marrow aspirate was layered gently onto 4 ml Ficoll (Ficoll Paque PLUS, Amersham Pharmacia Biotech UK Ltd, Little Chalfont, UK). This layered mixture was then centrifuged at 1510 rpm (400 g) for 30 minutes so that a straw coloured buffy layer formed in between the plasma and Ficoll erythrocyte residue. This buffy layer was recovered and washed by adding 10 ml Dulbecco's Modified Eagles Medium (DMEM, Sigma Aldrich, Poole, UK; 4500 mg/L glucose, L-glutamine and sodium pyruvate with 10% foetal calf serum, penicillin 50 iu/ml, and streptomycin 50 µg/ml). The sample was spun at 2000 rpm (702 g) for 10 minutes to remove heparin and Ficoll. The supernatant was discarded and the cell pellet resuspended in 12 ml DMEM. This cell suspension was then added to T75 flasks.

The primary seeded cells were allowed to adhere to the flask for two days before changing the medium, and thereafter the medium was changed every two days for 14-16 days when colony-forming units were visible. These cells were passaged before confluency by trypsinisation into T175 flasks and then expanded for a further 5-9 days until confluent.

Cells were removed from the flasks using trypsin digestion and centrifuged at 2000 rpm (702 g) for 10 minutes to pellet the cells. The medium supernatant was discarded and the cell pellet was resuspended in 1 ml of fresh DMEM without serum. A 20 µl aliquot was aspirated and counted in a haemocytometer to give the cell count per millilitre.

Quantification of MSCs in sequential aliquots of bone marrow:

To quantify the yield of MSCs from equine bone marrow, sequential 1.8 ml aliquots of bone marrow from three different horses (including the horse in which re-implantation was performed) were cultured separately. Cell numbers were determined after the colony-forming units were established, and at confluence after first passage.

Re-implantation of MSCs into superficial digital flexor tendinopathy:

6.4×10^5 cells (to give 40-50,000 cells/0.1 ml injected) were re-pelletted by centrifugation and then resuspended in 1.5 ml of platelet-rich plasma (PRP). The PRP was prepared from freshly obtained blood from the same horse by collecting 10 ml blood into sterile blood tubes containing 500 iu/ml heparin and centrifugation at 1620 g for 12 minutes. The top 2.5 ml of plasma was discarded and then 2.5 ml of PRP aspirated. This technique has been found to yield PRP with more than three times the number of platelets than normal plasma.

This 1.5 ml cell suspension was then injected into the damaged superficial digital flexor tendon of the same horse from which the cells were originally derived. The injection was performed in a sterile fashion under sedation and perineural analgesia at the proximal metacarpal site, in 15 x 0.1 ml (approximately 43,000 cells/0.1 ml) injections administered using a 23G,

1 inch needle along the length of the lesion from the palmar and medial aspects of the tendon while monitored ultrasonographically. The limb was then bandaged with a standard three-layered modified Robert Jones bandage.

Results

This protocol resulted in the generation of colony-forming units characteristic of MSCs in other species (Figure 1).

Quantification of MSCs in sequential aliquots of bone marrow:

The number of cells recovered before and after passage is shown in Table 1.

Table 1 – Quantification of MSCs from sequential bone marrow aliquots after *in vitro* culture.

Sample	Cell numbers from colony-forming units (subconfluent) (x 10^5)			Passage 1 (confluence) (x 10^5)		
Horse no.	1	2	3*	1	2	3*
Days culture	19	16	14	5	5	9
Peripheral blood (control)	-	0	-	-	-	-
Aliquot 1	31 (aliquots 1+2 combined)	9.0	-	78	61.4	-
Aliquot 2		21.4	-		57.8	-
Aliquot 3	-	2.2	-	-	44.6	45.2+
Aliquot 4	7.2 (aliquots 4+5 combined)	16.8	-	44.8	73	-
Aliquot 5		21.8	-		66	-
AVERAGE	12.2 (n=9)			47.1 (n=10)		

* = Horse used for implantation

\+ = Sample used for implantation

These cell numbers reflect the relative number of MSCs isolated in aliquots from the same horse as all samples were passaged at the same time. Approximately a million cells were obtained after initial culture for 14-16 days. In addition, it shows that passage will expand the cell numbers by a factor of between 2 and 20 times. No MSCs were cultured from a control sample of peripheral blood.

Injury characteristics

There was a central hypoechoic region in the superficial digital flexor tendon which occupied 45% of the cross-sectional area of the tendon at the maximum injury zone and extended from the mid to distal metacarpal region (levels 3-5 (Smith *et al*, 1994); 16-26 cm distal to the accessory carpal bone). The cross-sectional area of the tendon at the maximum injury zone was 64% larger than the contralateral tendon. The central lesion had already begun to fill with echogenic granulation/fibrous tissue (Figure 2).

Reimplantation of MSCs:

Accurate placement of the MSCs into the central tendon lesion was identified clearly from the air bubbles introduced at the time of injection. The injected cell/plasma mixture was observed to extend proximodistally to the limits of the lesion.

There was no observable swelling of the limb after the procedure. At re-examination, 10 days after implantation, there was no lameness at the walk and there was no increased thickening in the region of the superficial digital flexor tendon, although there was mild pain on digital pressure. Repeat ultrasonography showed no change in the substance of the tendon (Figure 2). Cross-sectional area measurements from all seven levels showed

minimal change from the re-implantation (average percentage change for all levels, 0.46% (decrease); maximum change at any one level, 9% (decrease)). There was thus no disruption to the tendon substance.

Discussion

This novel technique provides a method for the re-implantation of large numbers of autologous MSCs, which have been expanded in numbers *in vitro*, into the damaged tendon of the same horse. These cells have the potential to produce actual tendon matrix rather than poorly functional scar tissue, as occurs with conventionally managed superficial digital flexor tendinopathy. Equine superficial digital flexor tendinopathy, with its frequent centrally-positioned damage and surrounded either by relatively intact tendon tissue or thick paratendon (which invariably remains intact after even the most severe train-induced tendon injuries), in a tendon of sufficient size to make accurate intra-tendinous injection practical, lends itself perfectly as an enclosed vessel in which to implant MSCs. While the implantation of MSCs into other forms of damaged tendons and ligaments (eg eccentric lesions) may also prove to be beneficial, accurate placement, retention of cells, and minimising iatrogenic trauma caused by the injection process are more problematical, but may still be done, for example by using suspensions which gel *in situ*.

There was a larger variation in the cell numbers before than after passage because the cell numbers measured before passage were at sub-confluence and related to the number of colony-forming units on the plate derived from individual MSCs. Cell numbers at confluence after passage would be expected to be more constant because the cells expand until they cover the whole of the flask surface. Passage will therefore often be necessary to expand the numbers sufficiently.

An attempt was made to introduce approximately 50,000 cells/0.1 ml (approximately 500,000 cells in total). In view of the rapid expansion of cells *in vitro* after passage, it was expected that this number of cells would be sufficient to populate the central lesion in the tendon. Certainly, *in vitro* expansion of MSCs enables the autologous implantation of considerably larger numbers of MSCs than that available endogenously or delivered by direct injection of bone marrow, and avoids the potential adverse effects of other components of a bone marrow aspirate. In addition, storage of surplus cells frozen provides an additional source of MSCs if required subsequently.

Sufficient time had been allowed in this horse for adequate angiogenesis and granulation tissue to form which would be more likely to support MSCs than an earlier haemorrhagic lesion. Abundant growth factors are present in early healing tendon tissue (Cauvin, 2001) and the expanded MSCs were delivered in a platelet-rich plasma to augment this growth factor milieu.

This study has demonstrated that the first few millilitres of a bone marrow aspirate from the sternum can yield substantial numbers of MSCs after expansion in culture (in the order of 10^6 cells from 1.8 ml of bone marrow). The technique of equine MSC recovery from bone marrow, *ex vivo* culture and expansion, and re-implantation is both rational and feasible.

Example 2: Further detailed protocol for treating superficial digital flexor tendon or suspensory ligament injury

Criteria for inclusion of cases:

Superficial digital flexor tendon or suspensory ligament injury of the palmar aspect of the metacarpus which does not involve a tendon sheath. Only lesions with defined core lesions will be included and the current injury should be more than 3 weeks and less than 3 months in duration.

Protocol:

1) Baseline clinical examination to include full ultrasonographic examination and blood sample (for preparation of platelet-rich plasma and markers studies).

2) Cross-sectional areas of the damaged tendon to be calculated including tendon and lesion cross-sectional area for all seven transverse levels in the metacarpal region to give a percentage involvement of the lesion (severity).

3) After sedation (alpha 2 agonist and butorphanol), clipping and scrubbing over the sternum, individual sternebrae will be identified using diagnostic ultrasound and the inter-sternebral space marked on the skin with a sterile marker.

4) Local infiltration of local anaesthetic will be placed over the site for marrow aspiration (in centre of two adjacent sternebrae). A stab incision is made through the skin using a No 11 scalpel. A Jamshidi biopsy needle is introduced until it

hits to the sternebra. It is pushed a further 3-4 cm into the sternebra and then 2 x 2 ml aliquots of bone marrow from each of two sternebrae is aspirated into 2 ml syringes, pre-loaded with 500 iu (0.2 ml of 5000 iu/ml in each syringe) heparin.

5) After the aliquots have been obtained, a further 20 ml is withdrawn from one sternebra into a syringe pre-loaded with the same concentration of heparin (2 ml in 20 ml syringe). The bone marrow aspirate is then spun down at 2000 rpm for 20 mins and the supernatant collected, transferred to sterile 20 ml tubes, and frozen at -20°C.

6) 2 ml aliquots transferred into sterile 5 ml tubes.

7) Immediate transfer to Stanmore on ice.

8) Aliquots used for recovery and culturing of MSCs (see attached protocol on page 4).

9) Expansion of MSCs over approximately 1-2 week period until colonies of MSCs formed on plastic. Cells passaged and expanded further (for ~5 days until confluent) when there are approximately 7 x 10^6 cells/ml.

10) Cells removed from the flasks and divided into 3 aliquots.

11) Spun down in sterile tubes (1000 rpm for 10 minutes) to pellet the cells.

12) Aliquot 1 - used to characterize cells (ie ensure they are MSCs).

Aliquot 2 - cells frozen down in DMSO (for potential future use).

Aliquot 3 - prepared for injection.

13) Supernatant removed.

14) Cell pellet (approximately 7 x 10^6 cells) washed with fresh DMEMs without serum.

15) Spun down in sterile tubes (1000 rpm for 10 minutes) to pellet the cells.

16) Cells resuspended in 2 ml platelet-rich plasma (PRP) (or marrow supernatant), previously thawed, derived from the same horse.

17) Cells injected into the damaged tendon in a sterile fashion under sedation and perineural analgesia using multiple needle stabs (23G, 1 inch needle - 10 injections of 0.1 ml along the length of the lesion.

18) Limb bandaged with a standard modified Robert Jones bandage.

19) Tendons scanned at 3 days after injection and then the horse discharged from the hospital.

20) Tendons scanned at 3 days after injection and then the horse discharged from the hospital.

21) Horse is box-rested for 1 week and then given walking in hand exercise for a further 3 weeks before repeat ultrasound examination.

22) Repeat ultrasound examinations and blood samples at monthly intervals while following the controlled exercise programme shown below:

GUIDELINES FOR CONTROLLED EXERCISE PROGRAMME

Level	Minimum weeks after injury	Duration and nature of exercise
1	0-8	**30 minutes walking daily building this up to 45 minutes**
2	9-32	**walking + 5 minutes trotting building up to 30 minutes**
	9-12	40 minutes walking and 5 minutes trotting daily
	13-16	35 minutes walking and 10 minutes trotting daily
	17-24	30 minutes walking and 15 minutes trotting daily
	25-28	25 minutes walking and 20 minutes trotting daily
	29-32	15 minutes walking and 30 minutes trotting daily
3	33-52	**Walk and trot with restricted canter work**
	33-36	45 minutes exercise daily with slow canter up to 1 mile twice weekly
	37-40	45 minutes exercise daily with slow canter up to 1.5 mile twice weekly
	41-44	45 minutes exercise daily with one 3 furlong gallop three times a week
	45-48	45 minutes exercise daily with one 6 furlong

		gallop three times a week
	49-52	Increase exercise level gradually to full race/competition training
4	**From 52 weeks**	**Full race/competition training**

23) Compare results with an age-matched group of horses with similar lesions managed conservatively with the above exercise programme alone.

Outcome measures:

Ultrasonographic progression

Marker levels

Athletic outcome

If euthanased, tendon recovered for mechanical and matrix analyses.

Protocol for equine mesenchymal stem cell isolation from bone marrow

Materials

Ficoll	Marrow
5 ml syringe	pipette 12 ml x 3
Green syringe needle	pipettor
Universal	waste pot
Transfer pipette x 2	

FICOLL GRADIENT

1. Invert bottle of Ficoll to mix, snap off polypropylene cap, insert syringe through septum injecting air to equalise pressure. Invert bottle and withdraw 3 ml liquid.
2. Gently lay 4 ml bone marrow onto 30 ml Ficoll. The two layers are best achieved by holding the universal straight up and dispensing the marrow slowly down the side of the universal.
3. Centrifuge at 1510 rpm for 30 minutes (program 5 centrifuge in room 2 stops slowly and does not disturb layers) so that a straw coloured buffy layer forms in between the plasma and Ficoll erythrocyte residue.
4. Remove buffy layer to a fresh universal using a transfer pipette. Only mononuclear sells should be left in suspension.

SEEDING FLASKS

Materials

DMEM: 500 ml 4500 mg/L glucose, L-glutamine and sodium pyruvate
Foetal calf serum 10%, 50 ml
Penicillin 50 u/ml and Streptomycin 50 µg/ml

T75 x 2/T25 x 2
Waste pot
23 needle
5 ml syringe
12 ml pipettes x 3

5. Wash the buffy layer by resuspending the cells in 10 ml DMEM. Spin at 1500 rpm for 10 minutes to remove heparin and Ficoll.
6. Remove supernatant. Stem cells should be in the pellet.
7. Resuspend pellet in 2 ml DMEM using a 23 gauge syringe needle to give a single cell suspension.
8. Divide cells into two T75 flasks. T25s can be used if there was only a small volume of aspirate taken.

WASHING CELLS

9. Allow primary seeded cells to adhere to the flask for two days before changing the medium. (If setting up cells on Thursday, leave over the weekend.)
10. Change medium every two days.

OBSERVATIONS

- The flasks may appear cloudy. This is because there are erythrocytes in the suspension that will be washed off in subsequent DMEM washes.

- Stem cells can initially be observed as round shiny objects that have adhered to the flask unlike the surrounding cells in suspension.

- CFU-Fs should be seen after two weeks in culture.

NB 100-500 Human MSCs result from 50-100 million cells introduced into culture (Hayensworth S.E. *et al*).

Horse stem cells

Aim

Isolation and expansion of horse MSCs with a view to reinjecting the cells into the tendon.

Hypothesis

The number of cells in the initial 4 ml of aspirate extracted from horse marrow will yield a larger number of cells compared to the final 4 ml.

1. Marrow aspirate was taken from the horse sternum in the following aliquots (500 u/ml of heparin was used):

    1) Sample 1: 1-2 ml
    2) Sample 2: 3-4 ml
    3) Sample: 5-6 ml (given to horse)
    4) Sample 3: 7-8 ml
    5) Sample 4: 9-10 ml

2. Sample 1 and 2 were combined to give the first 4 ml of a 10 ml sample. Sample 3 and 4 were combined to give the final 4 ml of the 10 ml sample. They were named:

    - HS1 A: Sample 1+2
    - HS1 B: Sample 3+4

3. The technique for isolating stem cells from bone marrow outlined above was followed. Cells were passaged at into 2 x T75 flasks and were left in culture for 19 days.

4. Cell were counted and passaged into 2 x T175 flasks.

Cell count:

HSA1 P0 T75 .....3.1 x 10^6 cells/ml
HSB1 P0 T75 .....7.2 x 10^5 cells/ml

5. Cells were cultured for a 5 days until they reached confluency. Cells were counted at P1 and frozen down in DMSO.

Cell count:

HSA 1 P1 T175 .....7.8 x 10^6 cells/ml
HSB 1 P1 T175 .....4.48 x 10^6 cells/ml

Results

Cell count at P0:

HSA 1 P0 T75 .....3.1 x 10^6 cells/ml
HSB 1 P0 T75 .....7.2 x 10^5 cells/ml

Cell count at P1:

HSA 1 P1 T175 .....7.8 x 10^6 cells/ml
HSA 1 P1 T175 .....4.48 x 10^6 cells/ml

Conclusion

There is a higher yield of cells in the initial 4 ml of aspirate compared to the last 4 ml of marrow extracted

Example 3: Aftercare and controlled exercise programme after stem cell therapy

Injury: Superficial digital flexor tendonitis

Level	Minimum weeks after injury	Duration and nature of exercise
0	0-2	Box rest with bandaging
	Between weeks 1 and 2	**Repeat ultrasound examination at the RVC**
1	3-4	10 minutes walking in hand; maintain stable bandaging
	5-6	20 minutes walking in hand; maintain stable bandaging
	7-8	30 minutes walking in hand; maintain stable bandaging
	Between weeks 7 and 8	**Repeat ultrasound examination at the RVC**
2	9-12	40 minutes walking and 5 minutes trotting daily; can be ridden
	13-16	35 minutes walking and 10 minutes trotting daily
	17-24	30 minutes walking and 15 minutes trotting daily
	25-28	25 minutes walking and 20 minutes trotting daily
	29-32	15 minutes walking and 30 minutes trotting daily
	Between weeks 31 and 32	**Repeat ultrasound examination at the RVC**
3	33-36	45 minutes exercise daily with slow canter up to 1 mile twice weekly
	37-40	45 minutes exercise daily with slow canter up to 1.5 mile twice weekly
	41-44	45 minutes exercise daily with one 3 furlong gallop

		three times a week
	45-48	45 minutes exercise daily with one 6 furlong gallop three times a week
	49-52	Increase exercise level gradually to full race/competition training
	Between weeks 51 and 52	**Repeat ultrasound examination at the RVC**
4	From 52 weeks	Full race/competition training

The ultrasound re-examinations shown are the minimum number – further examinations can be performed as necessary.

This exercise programme may be altered (shortened or lengthened) depending on the progression of the case.

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CLAIMS

1. A method of treating a natural soft skeletal tissue injury in a patient the method comprising administering to the patient a composition of mesenchymal stem cells in liquid suspension enriched compared to the natural source of said cells.

2. A method according to Claim 1 wherein the injury is strain induced.

3. A method according to Claim 1 or 2 wherein the composition of mesenchymal stem cells is administered at the site of tissue injury.

4. A method according to any one of Claims 1 to 3 wherein the soft skeletal tissue is a tendon or ligament.

5. A method according to any one of the preceding claims wherein the patient is a mammal.

6. A method according to Claim 5 wherein the mammal is a human or a non-human mammal of economic importance.

7. A method according to Claim 6 wherein the non-human mammal is selected from the group consisting of horses, dogs and camels.

8. A method according to any one of the preceding claims wherein the patient is a horse.

9. A method according to any one of Claims 1 to 7 wherein the patient is a horse or a camel and the soft skeletal tissue is selected from the group consisting of superficial digital flexor tendon (SDFT),

suspensory ligament, deep digital flexor tendon, meniscus, cruciate ligament, and accessory ligament of the deep digital flexor tendon.

10. A method according to any one of Claims 1 to 7 wherein the patient is a dog and the soft skeletal tissue is selected from the group consisting of Achilles tendon, cruciate ligament, meniscus, flexor tendon and intervertebral disc.

11. A method according to any one of Claims 1 to 5 wherein the patient is a human and the soft skeletal tissue is selected from the group consisting of Achilles tendon, quadriceps tendon, rotator cuff, medial and lateral epicondylitis, cruciate ligament, meniscus and intervertebral disc.

12. A method according to any one of the preceding claims wherein the mesenchymal stem cells are allogenic.

13. A method according to Claim 12 wherein the mesenchymal stem cells are autologous.

14. A method according to Claim 13 wherein the mesenchymal stem cells are derived from the bone marrow of the patient.

15. A method according to any one of the preceding claims wherein the liquid suspension of mesenchymal stem cells is injected.

16. A method according to any one of the preceding claims wherein biological signals which encourage the mesenchymal stem cells to form tenocytes are also administered to the patient.

17. Use of a composition of mesenchymal stem cells in liquid suspension enriched compared to the natural source of said cells in the manufacture of a medicament for treating a natural soft skeletal tissue injury in a patient.

18. A kit of parts comprising (1) a composition of mesenchymal stem cells in liquid suspension enriched compared to the natural source of said cells, (2) means for delivering the liquid suspension of stem cells to a site of natural soft skeletal tissue injury in a patient and (3) means for determining that the means for delivering locate to the site of injury.

19. Any novel method as herein disclosed of treating a natural soft skeletal tissue injury in a patient.

ABSTRACT

A method of treating a natural soft skeletal tissue injury in a patient the method comprising administering to the patient a composition of mesenchymal stem cells in liquid suspension enriched compared to the natural source of said cells.

The method is particularly suited to the regeneration of tendons in competitive mammals, such as the superficial digital flexor tendon of the horse.

Figure 1.

Figure 1

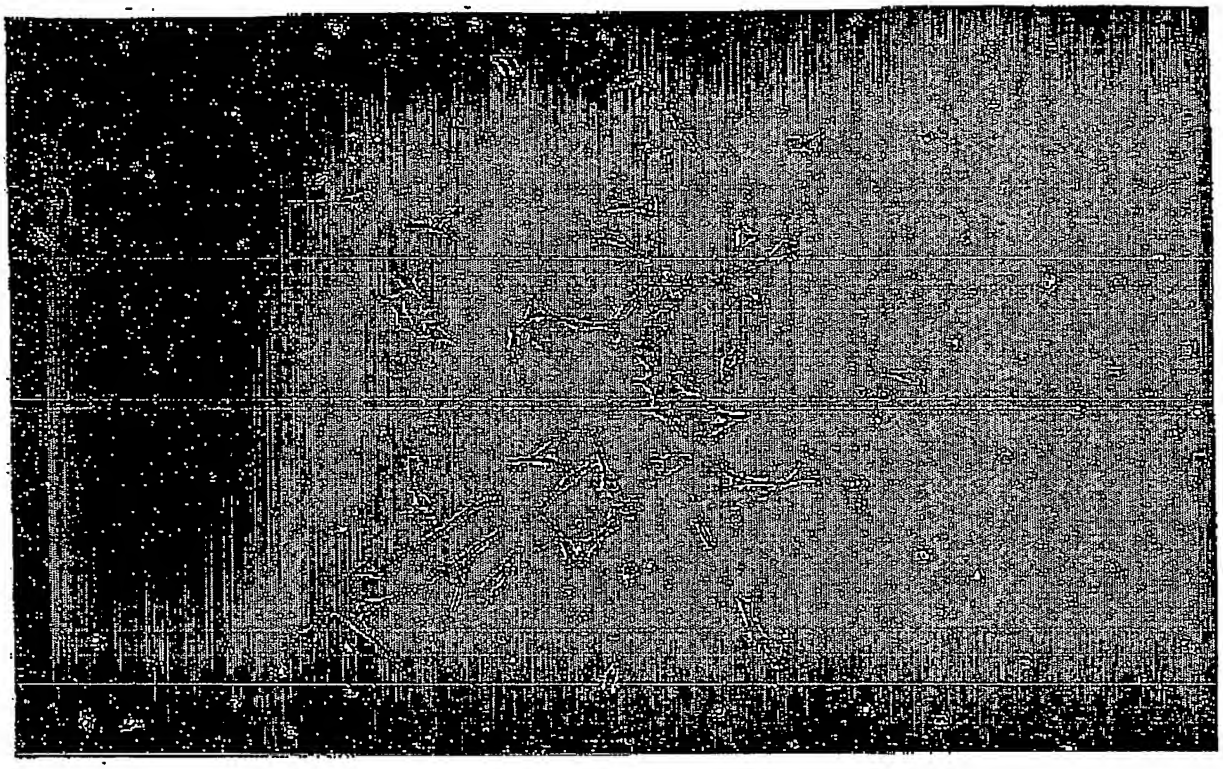
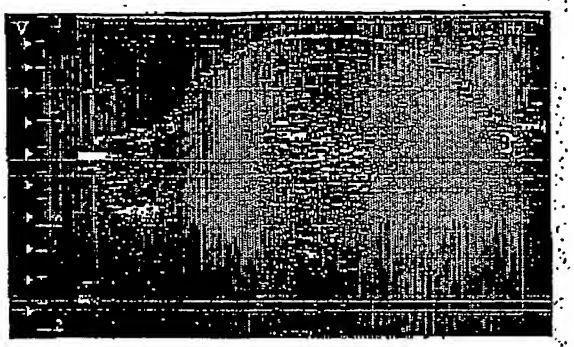


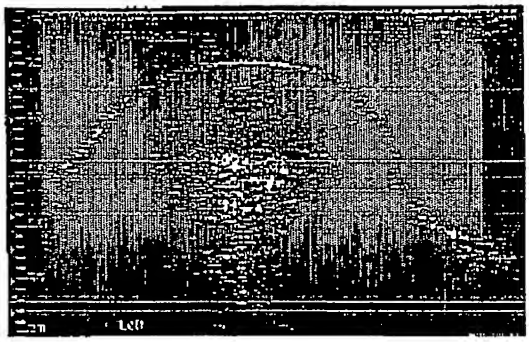
Figure 2

(a) Transverse image from level 4 (20cm distal to the accessory carpal bone)

(i) Before implantation

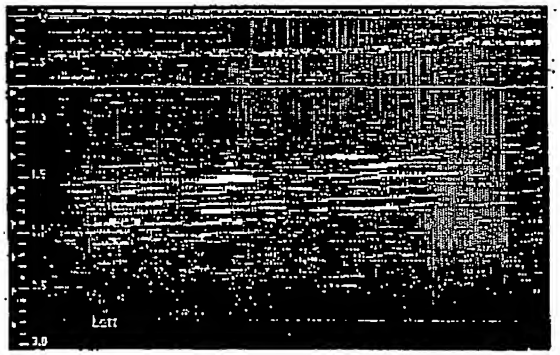


(ii) 10 days after implantation

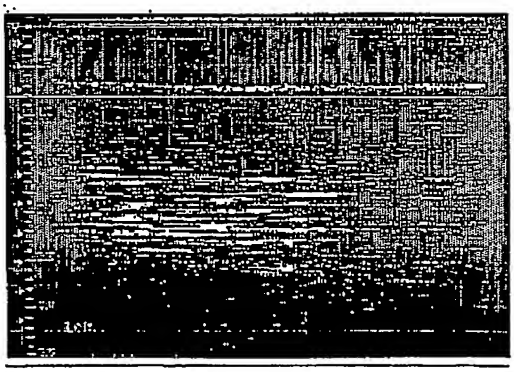


(b) Longitudinal image – 20-24cm distal to the accessory carpal bone

(i) Before implantation



(ii) 10 days after implantation



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